Ca^{2+} signals we developed a single-cell imaging approach that allows simultaneous monitoring of Cl^- channel activity and intracellular Ca^{2+} concentration in cultured dorsal root ganglion (DRG) neurons using simultaneous imaging of halide-sensitive H148Q/I152L EYFP mutant and fura-2. We investigated activation of Ca^{2+}-activated anion conductance in DRG neurons in response to i) Ca^{2+} release from the IP_3-sensitive intracellular stores induced by BK; ii) Ca^{2+} influx through the TRPV1 channels activated by capsaicin (CAP) and iii) Ca^{2+} influx via the voltage-gated Ca^{2+} channels (VGCC) induced by depolarization with extracellular solution containing 50 mM KCl (HK). Both BK and CAP produced large Ca^{2+} transients and caused significant quenching of YFP fluorescence. The onset of the YFP response to CAP was delayed as compared to the response to BK, possibly reflecting a shallower Ca^{2+} transient. Consistent with previous finding, activation of VGCC was least efficacious in activating CaCC and in approximately two thirds of neurons the YFP quenching was indistinguishable from the baseline rundown even despite strong Ca^{2+} transients produced by HK. Our results suggest that ANO1 in DRG neurons is preferentially coupled to colocalised Ca^{2+} sources.

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Phospholipase D2 Specifically Regulates TREK Channels via Direct Interaction and Local Production of Phosphatidic Acid

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Membrane lipids serve as second messengers and docking sites for proteins and play central roles in cell signalling. A major question about lipid signaling is whether diffusible lipids can selectively target specific proteins. One family of lipid-regulated membrane proteins is the TREK subfamily of K2P channels: TREK1, TREK2, and TRAAK. We investigated the regulation of TREK channels by phosphatidic acid (PA), which is generated by Phospholipase D (PLD) via hydrolysis of phosphatidylcholine. We found that, even though all three of the channels are sensitive to PA, only TREK1 and TREK2 are potentiated by PLD2 and that none of these channels is modulated by PLD1, indicating surprising selectivity. We find that PLD2, but not PLD1, directly binds to the C-terminus of TREK1 and TREK2, but not to TRAAK. The results lead to a model for selective lipid regulation by localization of phospholipid enzymes to specific effector proteins. Finally, by using the photoswitchable conditionnal subunit method to endow light sensitivity to the native TREK1 channels, we show that regulation of TREK channels by PLD2 occurs natively in hippocampal neurons.

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The Modulatory Function of the BK Channel γ1 Subunit is Determined by its Transmembrane Domain

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BK channels consist of pore-forming, voltage- and Ca^{2+}-sensing α-subunits (BKα), either alone or together with tissue-specific auxiliary β-subunits (β1-β4) or γ-subunits. The newly identified γ-subunits are a group of leucine-rich repeat (LRR)-containing membrane proteins which contain a single transmembrane (TM) segment, a short intracellular C-terminal tail (C-tail), an N-terminal signal peptide, and a relatively large extracellular LRR domain. The γ1 subunit (LRRC26), so far the most potent activator of BK channels, shifts the channel's voltage dependence of activation in the hyperpolarizing direction by ~140 mV. We investigated the role and mechanism of the γ1 TM domain in BK channel activation. We identified key amino acid residues in the γ1 hydrophobic TM region involved in BK channel activation. We found that a minimum of 3 positively-charged residues on the intracellular sides of TM segment are also required to maintain the γ1 subunit's full modulatory function, which likely act through stabilization of the TM domain for proper association with BK channels. We also found that the single TM segment and its capping charged residues can fully retain the γ1 subunit's BK channel-activating efficacy. We conclude that the TM domain is a major contributor to the potent channel-activating efficacy of the BK channel γ1 subunit.

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Differential Effects of PIP2 on Slo1 BK Channels with Different Auxiliary Subunits

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Phosphatidylinositol 4, 5-bisphosphate (PIP_2) regulates numerous ion channels, including large-conductance Ca^{2+}- and voltage-dependent K^+ (Slo1 BK) channels (Vaithianathan et al. 2008, J Gen Physiol 132:13-28). We examined the molecular and biophysical mechanisms of the effects of PIP_2 on human Slo1 BK channels with different auxiliary subunits heterologously expressed in HEK cells. In the absence of heterologously expressed auxiliary subunits, bovine brain PIP_2 (10 μM) applied to the cytoplasmic side inhibits currents through Slo1 channels. This inhibition is associated with a shift in GV by 18 mV and deceleration of the activation kinetics at positive voltages. In contrast, PIP_2 markedly increases currents through Slo1+β1 and hSlo1+β4 channels by shifting their GV curves by −46 mV and −34 mV, respectively. The stimulatory effect of PIP_2 on Slo1+β1 channels does not require Ca^{2+}-sensor activation or voltage-sensor activation. Currents through Slo1+β2 channels with a deletion in the N terminus of β2 to remove inactivation (Δ2-19) are not enhanced by PIP_2 but neutralization of 3 negatively charged residues in the β2 Δ2-19 N terminus introduces a modest effect of PIP_2. In Slo1+β1/β4, PIP_2 accelerates the macroscopic activation kinetics at positive voltages and decelerates the macroscopic deactivation kinetics at negative voltages, but in Slo1+β2 Δ2-32 PIP_2 has no effect on the deactivation kinetics. Measurements using chimeric β1-β2 subunits show that the second transmembrane domain and the C terminus of β1 is important for the large electrophysiological changes in Slo1+β1 channels caused by PIP_2. Furthermore, Slo1 ^{329}RKK^{331}, β1 R11, and β1 T14 are also critical to confer the effects of PIP_2. Supported in part by the NIH, DFG HE 2993/8, and Shanghai Science and Technology Commission.

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A Fluorogen-Activating Biosensor for Analysis of BK Channel Traffic and Surface Residency

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The regulation of ion channels is critical to numerous physiological processes, especially neuronal function. The large conductance voltage and calcium-activated potassium (BK) channel can modulate neuronal excitability, neurotransmitter release, and may be involved in the development of epilepsy; following a seizure, hippocampal BK channels are more highly trafficked to the plasma membrane. In addition to the pore-forming alpha subunits, which are sufficient to confer channel function, Beta subunits can be incorporated to alter gating and trafficking. Studies of the brain-specific Beta4 subunit effects on surface expression have produced conflicting results, likely due to alternative splicing in the alpha subunit. Fluorogen-activating peptides (FAPs) developed in our lab are well-suited to study protein trafficking; consisting of a ScFv-derived fusion tag and fluorogen dyes added to the cellular media, the association of these two cognate parts results in bright, specific fluorescence. By using a FAP-tagged BKα construct and a pair of fluorogen dyes, we have developed a method for two-color labeling of surface-resident and intracellular BK channels in live cells, allowing for rapid analysis of protein localization by flow cytometry and direct imaging. Using this system in stably expressing HEK293 cells, we found that modulation of the C-terminal region by kinase activity exerted strong changes in surface expression. Taken together with previous observations that these same kinases alter channel gating, this provides a synergistic model for BK-mediated hyperexcitability. A common confound in BK channel research is due to the vast number of splicing isoforms, each having different sensitivities to post-translational modifications. To that end, we are currently generating a knock-in mouse model in which our FAP-tagged BKα is included in all splice variants to examine trafficking changes *in Vivo* as well as analysis of the behavior of single channels.

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Understanding the Dynamics of K2P Channels in Complex Lipid Bilayers

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Ion channels are proteins that reside in phospholipid bilayers and conduct ions through their trans-membrane pore. Studying the dynamics of interaction between the channel, phospholipids, water and ions is fundamental to understanding ion channel function. We are using a combination of molecular dynamics simulations and functional tools to gain insight into K2P (Two Pore-domain Potassium Channel) function. By initially observing wetting and dewetting transitions in the inner pore of TWIK-1 (K2P1) channels during MD simulations and by subsequently combining in silico and in vitro mutagenesis and

electrophysiology, we have found a hydrophobic barrier deep within the inner pore of the TWIK-1 channel [1]. Our study suggests that this barrier contributes to the very low level of functional currents observed for TWIK-1 channels. We have also reviewed the computational, structural and functional evidence for hydrophobic gating in several ion channel families and propose that understanding the dynamic behavior of water and ions within the pore represents an increasingly important element in understanding the relationship between ion channel structure and function [2]. We are now examining the interaction between K2P channels and phospholipids in more detail. Using MD simulations, we find hot spots for K2P channel and lipid interactions. These findings suggest that lipids can play modulatory roles in K2P channel function.

[1] Aryal P, Abd-Wahab F, Bucci G, Sansom MSP & Tucker SJ. A hydrophobic barrier deep within the inner pore of the TWIK-1 K2P potassium channel. Nature Communications 5:4377 (2014) [http://dx.doi.org/10.1038/ncomms5377]

[2] Aryal P, Sansom MSP and Tucker SJ. Hydrophobic Gating in Ion Channels. Journal of Molecular Biology (2014) [http://dx.doi.org/10.1016/j.jmb.2014.07.030]

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Non-Markovian Protein Dynamics in a Near-Critical Membrane Model

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Recent experiments in Giant Plasma Membrane Vesicles isolated from living cells have suggested that cell membranes are tuned close to a liquid-liquid critical point. Perturbations which influence membrane criticality - anesthetics and cholesterol level modulators - also affect the functioning of a large number of membrane-bound receptors and ion channels. This motivates us to develop a model for a membrane-bound protein that is allosterically regulated by interactions with its surrounding near-critical membrane. We consider a two dimensional lattice where Ising spins represent membrane lipids, and a two-state protein is represented as a group of like spins that must flip together. In our model, the full state of the system, including both protein and membrane degrees of freedom, obeys Markovian dynamics. However, when the protein is considered in isolation, as is typical experimentally, its dynamics become non-Markovian. We show that this phenomenon arises as information about the past state of the protein is stored in membrane degrees of freedom. Our model suggests a unified mechanism underlying the susceptibility of various ion channels to both anesthetics and cholesterol modulation and presents a new role for membrane lipids in the collective allosteric regulation of proteins.

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Mechanisms of TREK-2 Potassium Channel Gating

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The K2P channel TREK-2 is an archetypal polymodal potassium channel which acts to couple a diverse range of regulatory stimuli to cellular electrical excitability. Alongside the other thermo-and mechano-gated K2P channels (TREK-1 and TRAAK) the TREK-2 channel is critical for discrimination of innocuous and noxious temperature and touch sensation. Guided by novel X-ray crystal structures, we have used a variety of electrophysiological, pharmacological and kinetic studies to demonstrate a mechanism of action for the state-dependent inhibition of TREK-2 by norfluoxetine, a biologically active metabolite of the anti-depressant Prozac. These studies also enable us to propose a structural basis for activation of TREK-2 by membrane stretch, temperature and arachidonic acid.

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The Molecular Basis for Heme Modulation of KATP Channels

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Heme, iron protoporphyrin IX, is a vital prosthetic component of a number of functional hemoproteins playing essential roles in a diverse range of biological actions including oxygen transport, electron transport, catalysis and gene regulation. An emerging role of heme is its ability to regulate the activity of ion channels including voltage gated K^+ (Kv), large conductance Ca^{2+}-activated K^+ (BK) and epithelial Na^+ (ENaC) channels. Here we report heme regulation of the ATP-sensitive K^+ (KATP) channel in both cardiac myocytes and HEK293 cells heterologously expressing Kir6.2 and SUR2A subunits. The KATP channel is sensitive to the intracellular nucleotides ATP and ADP. The KATP channel links cellular metabolic state and excitability most notably during ischaemic stress. ATP acts with high affinity upon the cytosolic face to inhibit opening, thus the channel opens during periods of depleted cellular energy.

Whole-cell KATP currents of both ventricular myocytes and HEK293 cells expressing KATP channels were increased upon application of 500 nM hemin extracellularly. In inside-out patches, KATP channel activity was reduced in the presence of ATP (500 μM), but on subsequent addition of hemin (500 nM) KATP channel open probability significantly increased from 0.024 ± 0.013 to 0.110 ± 0.028 ($n = 6$, $P < 0.01$).

Sequence alignments with known heme binding regions revealed a structurally unresolved region located on an intracellular linker in the non-pore forming SUR2A subunit containing the residues C628XXH631 and H648, which was analogous to the reported Kv1.4 heme binding sequence. Mutating these residues (C628S, H631A and H648A) led to reduced sensitivity of the resulting KATP channels to heme. Mutagenesis of each residue revealed C628 and H648 to have the greatest effect at reducing the agonistic effects of heme. Here we provide evidence for heme binding and regulation of KATP

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NMR Structural Studies of the Binding of Activating Mamba Toxin Tx7335 on the Potassium Channel KcsA

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We have recently identified a novel 63 amino acid residue three-finger toxin (called Tx7335) from eastern green mamba snake (Dendroaspis angusticeps) which interacts with KcsA and induces an increase in frequency and duration of individual channel openings when added to the outside of the channel. The toxin exerts this activating effect both on wild-type KcsA as well as on an agitoxin2-sensitive mutant form of the channel, indicating a mode of action and binding site that are different from the classic pore-blocker toxins. We are currently using NMR spectroscopy to unravel the structural underpinnings of this mechanism of action. High yield of purified 15N labeled KcsA and excellent NMR spectral quality have been achieved. Currently the characterization of toxin binding using 1H15N correlation spectra of 15N labeled KcsA in the absence and presence of toxin is ongoing. Experiments are conducted in different membrane mimetics including DMPC/DHPC bicelles and DPC or DM micelles at different pH, temperature and salt concentration. Some chemical shifts and peak intensity changes upon toxin addition have been observed. Continuing NMR structural studies will further elucidate the mechanism of how Tx7335 interacts with KcsA and shed light on the conformational and dynamic changes of C-type inactivation in KcsA and on a novel mechanism of ion channel regulation.

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Mechanism of Inhibition of the GluA1 AMPA Receptor Channel Opening by 2,3-Benzodiazepine Compounds

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2,3-Benzodiazepine derivatives, also known as GYKI compounds, represent a group of the most promising synthetic inhibitors of AMPA receptors. Here we investigate the mechanism of inhibition of the GluA1 channel opening and the site of inhibition by GYKI 52466 and its N-3 methyl-carbamoyl derivative (BDZ-*f*) as well as two N-3 thiadiazolyl compounds. Like GluA2, GluA1 is a key AMPA receptor subunit, and excessive activity of GluA1 has been implicated in a number of neurological disorders. Using a laser-pulse photolysis technique, we investigated the mechanism of inhibition of the GluA1 channel expressed in HEK-293 cells. We found that these compounds inhibit the GluA1 channel noncompetitively. Addition of a methyl-carbamoyl group or a thiadiazole moiety to the N-3 position of the diazepine ring with the azomethine feature improves the potency of the resulting compounds without changing the site of binding, which we termed as the ''M'' site. On the basis of the magnitude of the inhibition constants for the same inhibitors (i.e., GYKI 52466 and BDZ-*f*), the ''M'' sites on GluA1 and GuA2 are different. Overall, the ''M'' site on GluA2 accommodates the same compounds better, or the same inhibitors show stronger potency on GluA2 than GluA1, if the N-3 pocket is not fully occupied. Acylating the N-3 position to occupy the N-3 side pocket of the ''M'' site can significantly narrow the difference and improve the potency of a resulting compound on GluA1. The two thiadiazolyl benzodiazepines inhibit both GluA1 and GluA2 much more strongly and almost equally potently. A thiadiazole moiety is thought to occupy more fully the side pocket of the ''M'' site, thereby generating a stronger, multivalent interaction between the inhibitor and the receptor binding site. *This work is supported by NIH/NINDS.*